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Drugs that inhibit microtubule functions are one of many anti-neoplastic drugs that are used to combat breast and other cancers. Taxol and vincristine are microtubule poisons that block that proper function of microtubules that are essential for a broad spectrum of motile biological processes that include cell division, vesicle transport, cell shape, and flagella functions. For rapidly proliferating cancer cells, anti-microtubule drugs offers a highly effective means to block cell division and thus stop tumor growth. Nevertheless, these drugs block other microtubule dependent processes that adversely affect the functions of many non-dividing cells. Furthermore, there is the complication that the cancer cells can develop multi-drug resistance that makes them refractile to conventional antineoplastic agents. The identification of novel drugs with increased selectivity towards mitotic processes and act synergistically with existing anti-microtubule drugs should enhance and refine the modalities used to treat breast cancer patients. Our interest in the molecular and biochemical mechanisms that are central to mitosis in human cells has led to the identification of novel proteins and pathways that are suited for designing highly specific anti-mitotic drugs. The objective of this proposal is to disrupt such pathways in established breast cancer cell lines to validate them as suitable targets for developing new anti-mitotic drugs.

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Validate Mitotic Checkpoint and Kinetochore Motor Proteins in Breast Cancer Cells as Targets for the Development of Novel Anti-Mitotic Drugs.

Introduction:

Drugs that inhibit microtubule functions are one of many anti-neoplastic drugs that are used to combat breast and other cancers. Taxol and vincristine are microtubule poisons that block the proper function of microtubules that are essential for a broad spectrum of motile biological processes that include cell division, vesicle transport, cell shape, and flagella functions. For rapidly proliferating cancer cells, anti-microtubule drugs offers a highly effective means to block cell division and thus stop tumor growth. Nevertheless, these drugs block other microtubule dependent processes that adversely affect the functions of many non-dividing cells. Furthermore, there is the complication that the cancer cells can develop multi-drug resistance that makes them refractile to conventional anti-neoplastic agents. The identification of novel drugs with increased selectivity towards mitotic processes and act synergistically with existing anti-microtubule drugs should enhance and refine the modalities used to treat breast cancer patients. Our interest in the molecular and biochemical mechanisms that are central to mitosis in human cells has led to the identification of novel proteins and pathways that are suited for designing highly specific anti-mitotic drugs. The objective of this proposal is to disrupt such pathways in established breast cancer cell lines to validate them as suitable targets for developing new anti-mitotic drugs.

Body:

We proposed to manipulate two pathways that are known to be essential and operate only in mitosis of human cells to validate them as suitable targets for the development of novel anti-neoplastic agents. One pathway is specified by the kinesin-like motor protein CENP-E that is essential for aligning chromosomes at the spindle equator during mitosis. The second pathway is a checkpoint pathway that is specified by multiple proteins to ensure cells do not prematurely exit mitosis in the presence of unaligned chromosomes. We proposed four tasks to achieve our goals. We have chosen to analyze three established breast cancer lines and compare their responses to the Hela cervical carcinoma cell line, with which we have studied these two pathways extensively.

Task 1. Evaluate expression of mitotic proteins CENP-E and checkpoint proteins in established breast cancer lines.

We have conducted immunoblot analysis to determine the expression of CENP-E and the checkpoint proteins, hBUB1, hBUBR1, MAD1, MAD2 and Cdc20 in MCF7, T47D and MDA468 cells. All of these proteins were found to be expressed in these cell lines and thus confirmed that they are valid in vivo targets (data not shown). We have determined that all of these proteins are localized to kinetochores in MCF7 and MDA-468 cells. Figures 1 and 3 show localization of hBUB1 and CENP-E to kinetochores of mitotic MCF7 and MDA468 cells, respectively. The presence of CENP-E and various checkpoint proteins at kinetochores support our prediction that these proteins provide similar functions in mitosis as we have shown in Hela cells.

We have also examined the response of MCF7 and MDA468 cells to the microtubule inhibitor, nocodazole and found that this drug will delay cells in mitosis. These findings indicate that the mitotic checkpoint pathway is likely to be intact in these cancer cell lines. Thus, the various checkpoint proteins that we proposed to analyze in this project are strong candidates with which we can use to inhibit this pathway.

Our studies of T47D are lagging because these cells grow at only half the rate as the other cell lines. This unavoidable technical problem has prevented us from conducting all of our studies simultaneously. We therefore intend to continue to study this cell line independently of MCF7 and MDA468.

Task 2. Evaluate response of T47D, MCF-7 and MDA-MB-468 cells to inhibition of the mitotic checkpoint proteins, hBUBR1, hBUB3, cdc20 and MAD2.

As we have confirmed that these breast cancer lines express the target mitotic checkpoint proteins, we have initiated efforts to inhibit the mitotic checkpoint. We had originally proposed to accomplish this by microinjecting antibodies and overexpression of dominant negative mutants. However, new advances in silencing gene expression by RNA interference (RNAi) have altered our original strategy.

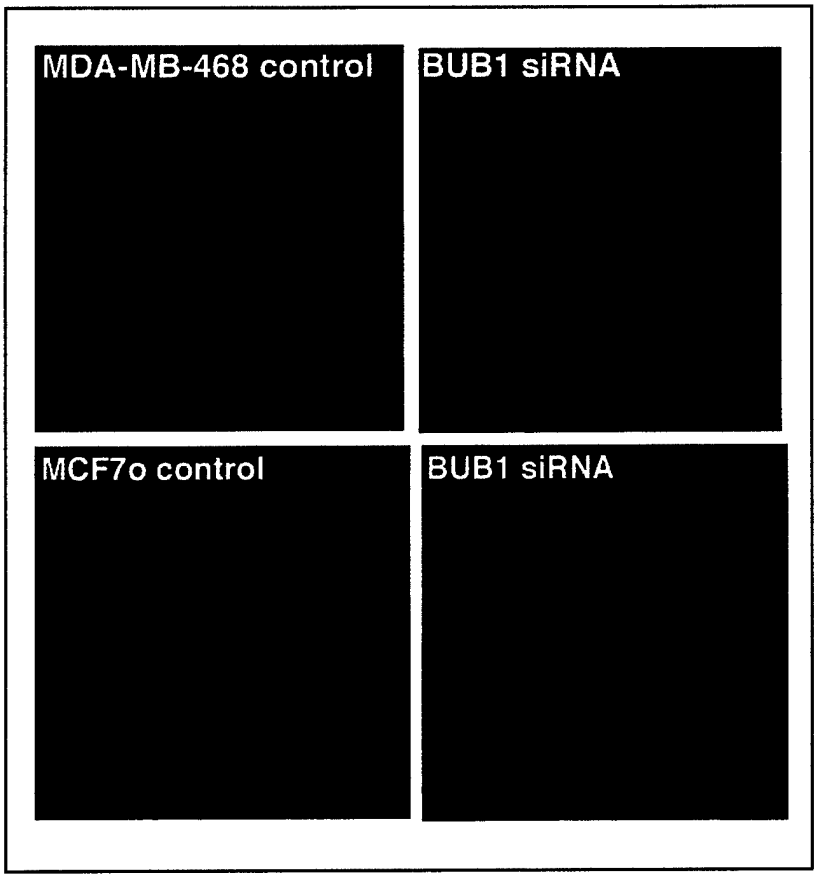

Figure 1. MDA468 and MCF cells were trans-fected with a control (left panels) or BUB1 (right panels) siRNAs and mitotic cells were stained with rabbit anti-hBub1, mouse anti-tubulin and DNA. Images were captured with a 63X oil objective and then pseudo-colored and merged.

Using siRNA, we have successfully inhibited the expression of hBUB1 kinase in Hela cells. One unexpected finding was that the loss of hBUB1 prevented the assembly of MAD1, MAD2 and hBUBR1 checkpoint proteins to the kinetochore (data not shown). Thus, inhibition of hBUB1 kinase may result in the inhibition of multiple checkpoint proteins. Based on these studies, we have transfected MCF7 and MDA468 cells with hBUB1 siRNA. At the single cell level, it is clear that hBUB1 expression can be reduced by siRNA (Figure 1). However, the low transfection efficiencies of these cell lines have made it difficult to interpret results from clonogenic experiments. While there are instances where cells transfected with hBUB1 siRNA exhibited reduced efficiency of colony formation (Figure 2), this outcome is highly variable.

We attribute this to the variability in transfection efficiencies of MCF7 and MDA468 cells. To overcome this obstacle, we plan to infect cells with a recombinant lentivirus that express the siRNA of interest. This viral delivery system was developed to overcome problems with poor transfection efficiencies. We are in the process of making the appropriate constructs so that we may generate large stocks of recombinant lentivirus for the clonogenic studies.

Figure 2. MCF7 (top panel) and MDA-468 (bottom panel) cells were transfected with BUB1 (left), control (center) and CENP-E (right) siRNAs and were plated at approximately 200 cells per 35cm plate. Colonies were stained and counted on day 15 for MCF7 and day 11 for MDA468. Left axis represents colony number.

Task 3. Evaluate CENP-E as a target to block T47D, MCF-7 and MDA-MB-468 cells in mitosis.

As with our studies of the checkpoint pathway, we have opted to inhibit CENP-E function by RNAi technology. Using Hela cells as a positive control, we succeeded to inhibit expression of CENP-E and cells arrest in mitosis because chromosomes fail to align properly (data not shown). As before, we are able to reduce CENP-E expression in MCF7 and MDA468 cells at the single cell level. We are therefore also generating recombinant lentivirus that express CENP-E RNAi so that we may conduct our clonogenic survival studies.

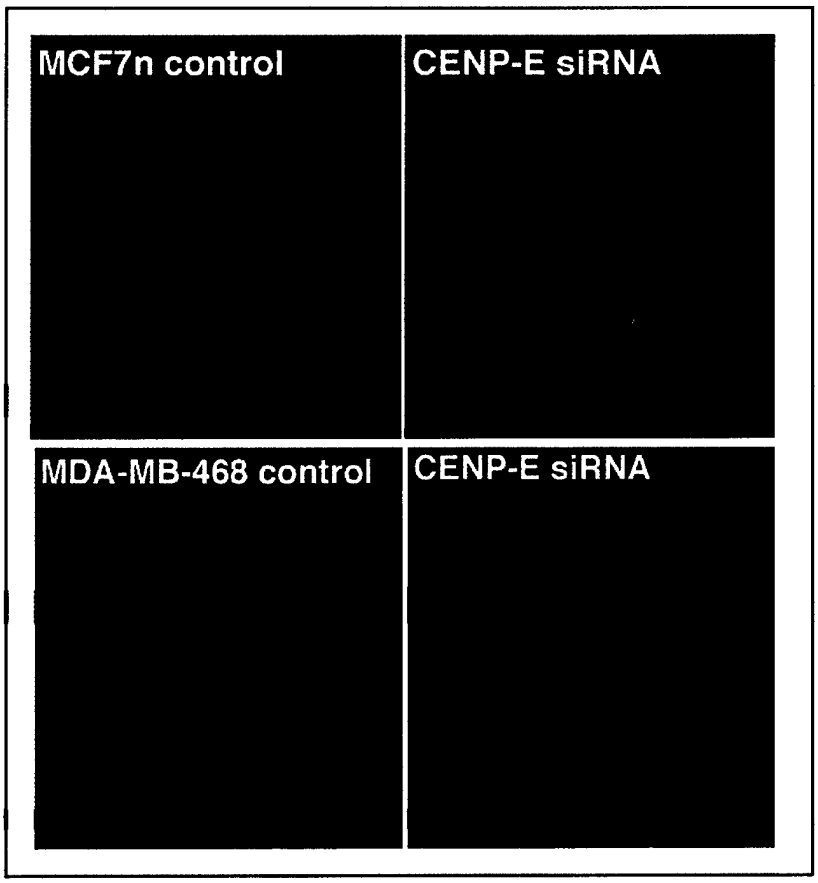

Figure 3. MDA468 and MCF cells were trans-fected with a control (left panels) or CENP-E (right panels) siRNAs and mitotic cells were stained with rabbit anti-CENP-E, mouse anti-dynein and DNA. Images were captured with a 63X oil objective and then pseudo-colored and merged.

Task 4. Maintaining stocks of affinity purified antibodies.

Over the past year, we have generated monoclonal antibodies to hBUB1, hBUBR1 and MAD1 proteins. The existence of monoclonal antibodies to these and other checkpoint proteins provides us with a continuous source of high quality antibody. While the efforts to generate monoclonal antibodies are significant, we are certain that it will reduce the labor that is required to maintain stocks of polyclonal antibodies. We have recently generated a monoclonal antibody against CENP-E. We did not succeed to generate any Mad2 monoclonal antibodies. We will continue to rely on polyclonal antibodies for those proteins that we do not have monoclonal antibodies against.

Key Research Accomplishments:

- Confirmed expression and localization of CENP-E and the mitotic checkpoint proteins hBUB1, hBUBR1, MAD1, MAD2, Cdc20 and CENP-E, in MCF7 and MDA468 cells.
- Verified that siRNA can inhibit the expression of CENP-E and hBUB1 in MCF7 and MDA468 cells.
- Generated monoclonal antibodies to hBUB1, hBUBR1,MAD1 and CENP-E.

Reportable Outcomes:

Jablonski, SA., Liu, ST., Yen, TJ. Targeting the Kinetochore for Mitosis-Specific Inhibitors. *Cancer Biology and Therapy* 2:236-241. 2003.

Liu, St., van Deursen, J., Yen TJ., The role of the mitotic checkpoint in maintaining genomic stability. *Current topic in Developmental Biology.* G. Schatten Ed. *In press.*

Licensed hBUB1 and hBUBR1 monoclonal antibodies to BD Sciences.

Conclusions:

We validated the expression of candidate target genes in various breast cancer lines and have used siRNA to inhibit their expression in these cells. However, the low transfection efficiencies has prevented us from conductiong clonogenic survival experiments. Nevertheless, we are optimistic that the viral delivery system will allow populations of cells to be uniformly infected with recombinant lentivirus that express siRNA. This will allow us to reliably evaluate results from clonogenic studies.

Appendix:

Jablonski, SA., Liu, ST., Yen, TJ. Targeting the Kinetochore for Mitosis-Specific Inhibitors. *Cancer Biology and Therapy* 2:236-241. 2003.

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Review

Targeting the Kinetochore for Mitosis-Specific Inhibitors

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mitosis, kinetochore, drug target

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ABSTRACT

Microtubule poisons such as taxol and vinblastine are widely used to treat a variety of cancers. These drugs are believed to kill cells by blocking mitosis. However, there is a critical need to identify new drugs because tumors can often become refractory to treatment with existing drugs. Studies over the past decade on chromosome segregation have uncovered a plethora of novel proteins that function specifically in mitosis. Centrosomes and kinetochores are two organelles that specify formation of the spindle and the attachment of chromosomes to the spindle, respectively. The focus of this review is to highlight the kinetochore as a rich source of targets for the development of mitosis-specific drugs.

INTRODUCTION

Microtubules are dynamic polymers that support a variety of cellular functions such as vesicle and organelle transport, cell shape and polarity, beating cilia, and spindle functions in mitosis. The latter function is the most desirable target for cancer chemotherapy. Indeed, the use of anti-microtubule drugs such as paclitaxel, docetaxel and the vinca alkaloids for the treatment of a variety of cancers is based on the ability of these drugs to inhibit mitosis and thus cell proliferation.[1] As these drugs inhibit other essential microtubule-based processes, they cannot specifically target mitotic cells. Of greater concern however is the high frequency at which cancer cells develop resistance to these drugs.[2] Cancer cells frequently become resistant as a result of elevated P-glycoproteins, alterations in the levels of tubulin isotypes or mutations in the tubulin subunits. For these reasons, the need to identify new drugs that inhibit the proliferation of cancer cells is imperative.

The kinetochore is a macromolecular complex that was first described at the EM level as a trilaminar disc that is situated on opposite sides of the highly condensed centromeric heterochromatin.[3-5] This structure is only visible in mitosis and therefore suggests that its assembly is a cell-cycle regulated event. For the purposes of this review, we define centromeres as the cis-acting DNA sequences that specify the assembly of a constellation of proteins that are considered to form the kinetochore (Table 1). Amongst these are proteins that specify microtubule attachments and checkpoint functions. Separately, there is a large number of proteins that are thought to play a structural role by contributing to the formation of a highly organized trilaminar kinetochore.[4,5] As many of these proteins are believed to function exclusively in mitosis, they are ideal candidates for drug discovery (Fig. 1).

KINETOCHORE MICROTUBULE ATTACHMENTS

One of the most important kinetochore functions in mitosis is establishing connections between the spindle and chromosomes.[6] Anti-microtubule drugs inhibit mitosis by interfering with the attachement of chromosomes to the spindle. However, direct inhibition of kinetochore proteins that are important for establishing microtubule connections exclusively in mitosis has the advantage of selectively targeting rapidly dividing cells. Kinetochores of vertebrate cells contain three known microtubule-based motors that include dynein, and the kinesin-like proteins, CENP-E and MCAK.[7-10] In addition, microtubule binding proteins such as CLIP170, EB1, and CLASP/Orbit are also present at kinetochores where they are also likely to mediate microtubule interactions with the kinetochore.[11-13] Given that the molecular motors are ATPases, they are better suited for drug development as it is possible to use the existing libraries of ATP analogs to screen for a suitable inhibitor. As dynein function is required in a wide variety of cellular processes besides mitosis, compounds that directly inhibit dynein will not achieve the desired specificity. On the other hand, CENP-E and MCAK are suitable candidates because they appear to be only critical for mitosis. Cells depleted of CENP-E fail to align their chromosomes properly and arrest in

mitosis despite the formation of a normal looking bipolar spindle.[14,15] As the motor domain of CENP-E has been shown to be essential for its function, it may be possible to identify inhibitors by screening for compounds that inhibit its ATPase activity. Although the effect of disrupting CENP-E on cell killing remain unknown, it is clearly an essential gene in mouse.[16] Given its importance in mitosis, it is not surprising that CENP-E knockout mice die during early stages of embryogenesis. These obser - vations strongly suggest that disruption of CENP-E will cause cells to die.

MCAK is an unconventional kinesin because it does not behave as a classic motor that translocates along the microtubule lattice. Instead, MCAK and its frog homolog, XKCM1, induce microtubule shortening.[17,18] At kinetochores, MCAK is believed to stimulate the depolymerization of the attached microtubules so that chromosome can move towards the poles. Thus, cells disrupted of MCAK accumulate lagging chromosomes in anaphase that is consistent with the biochemical properties of this protein. The presence of lagging chromosomes implies that these cells will divide and become aneuploid. One likely outcome is that many of the aneuploid progeny cells die.

TARGETING THE MITOTIC CHECKPOINT

The mitotic checkpoint is an evolutionarily conserved mechanism that prevents cells with even a single unaligned chromosome from exiting mitosis.[19] Cells with unaligned chromosomes that override the checkpoint will divide and produce aneuploid cells. Given that the majority of aneuploid cells will die because of massive chromosome imbalance,[20,21] the mitotic checkpoint should in principle be a reasonable target for drug development. It is clear that disruption of kinetochore proteins such as CENP-E or the spindle with conventional anti-microtubule drugs will arrest cells in mitosis. What is less clear is how these cells eventually die. One possibility is that cells die as a result of overriding the checkpoint. The biochemical activities of checkpoint proteins that arrest cells in mitosis cannot be sustained indefinitely. Thus, checkpoint arrested cells eventually will exit mitosis regardless

Table 1 **CENTROMERE/KINETOCHORE PROTEINS FROM YEAST TO HUMANS**

The known human centromere/kinetochore proteins are classified into seven categories. Not all the centromere/kinetochore proteins in model organisms are shown here. Different names of the same protein are separated by slash (/). Different proteins are separated by comma (,). The classification is not very strict, and some proteins have been put into more than one category. The references on individual proteins can be provided on request.

(A) Constitutive Centromere Proteins—Localized at Centromeres Throughout the Cell Cycle

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
CENP-A	Cse4	CENP-A/Cnd1	Cid	HCP3	CENP-A
CENP-B	Cbf1	Abp1, Cbh1, Cbh2			
CENP-C	Mif2			HCP4	
CENP-D					
CENP-G					
CENP-H		Sim4			
CENP-I/LRPR1	Ctf3	Mis6			
hMIS12	Mtw1	Mis12			

(B) Regulatory Proteins—Mitotic Checkpoint Proteins and Proteins Involved in Mitotic Regulation

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
MAD1	Mad1	Mad1		MDF1	xMAD1
MAD2	Mad2	Mad2		MDF2	xMAD2
BUBR1	Mad3,Bub1	Mad3			xBUBR1
BUB1	Bub1	Bub1	BUB1	CeBUB1	xBUB1
BUB3	Bub3	Bub3	BUB3		xBUB3
MPS1/TTK	Mps1	Mph1			xMPS1
hZW10			ZW10	CeZW10	ZW10
hROD			ROD	Ce ROD	ROD
RAE1					
CDC20/p55	Cdc20	Slp1	Fizzy/Fzy	FZY1/Fizzy	CDC20
APC1	Apc1	Cut4		MAT-2	APC1/BimE
APC3/ CDC27	Cdc27	Nuc2		MAT-1	APC3/CDC27
APC10/DOC1	Apc10/Doc1	Apc10			APC10
PLK1	Plk1/Cdc5	Plo1	POLO		Plx1

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(C) Microtubule Motors or Associated Proteins Whose Kinetochore Localization is Independent of Mictrotubules

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
CENP-E			CENP-meta, CENP-ana		CENP-E
Dynein/dynactin			dynein		
MCAK				CeMCAK	XKCM1
hZW10			ZW10	CeZw10	xZW10
hROD			ROD	CeROD	xRod
hZWint-1			Zwint-1		
hZWilch			Zwilch		
CLIP-170	Bik1	Tip1	D-CLIP-190		
CLASP	Stu1		MAST/Orbit		
HEC1	Ndc80	Ndc80			xNDC80
hNuf2	Nuf2	Nuf2		HIM10	xNUF2

(D) Microtubule Associated Proteins Whose Kinetochore Localization is Dependent Upon Mictrotubules

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
LIS1	Pac1				
APC					
EB1	Bim1	Mal3			
ch-TOG	Stu2	Dis1, Mtc1/Alp4			XMAP215

Table continued on next page.

Table 1 **CENTROMERE/KINETOCHORE PROTEINS IN HUMAN AND SEVERAL MAJOR MODEL ORGANISMS, CONT.**

The known human centromere/kinetochore proteins are classified into seven categories. Not all the centromere/kinetochore proteins in model organisms are shown here. Different names of the same protein are separated by slash (/). Different proteins are separated by comma (,). The classification is not very strict, and some proteins have been put into more than one category. The references on individual proteins can be provided on request.

(E) Chromosomal passenger Proteins

Human	S. cerevisiae	S. pombe	Drosophila	C. elegans	Xenopus
Aurora-B/ AIRK2	Ipl1	Ark1	Aurora B	AIR-2	AuroraB
Survivin	Bir1	Cut17/Bir1	survivin	ICP-1, ICP-2?	survivin
INCENP	Sli15	Pic1	INCENP	INCENP	INCENP
TD60					

(F) Nuclear Pore Proteins

Human	S. cerevisiae	S. pombe	Drosophila	C. elegans	Xenopus
RanGAP					
Nup358/RanBP2					
Nup133					
Nup105					
MAD1	MAD1				xMAD1
MAD2	MAD2				xMAD2
MPS1/TTK					

(G) Structural Proteins/Unknown Functions

Human	S. cerevisiae	S. pombe	Drosophila	C. elegans	Xenopus
CENP-F				HCP1, HCP2	
53BP1					53BP1
Topollα					
PARP1					
cohesin					
hRAD21/hSCC1	Mcd1/Scc1/Rad21	Rad21			xSCC1
STAG1/SA2	Scc3	Psc3			
STAG2/SA2	Scc3				
STAG3	Scc3				
hSMC1	Smc1	Psm1/Smc1			xSMC1
hSMC3	Smc3	Psm3/Smc3			xSMC3
hPDS5	Pds5	Pds5			
condensin					
hCAP-E/SMC2	Smc2	Cut14		MIX1	xCAP-E
hCAP-C/SMC4	Smc4	Cut3		SMC4	xCAP-C
hCAP-H/BRRN	Brn1	Cnd3	BARREN		xCAP-H/ Barren
hCAP-G	Ycs4	Cnd2			xCAP-G
hCAP-D2/ CNAP1/hEg7		Cnd1			xCAP-D2/ pEg7

of whether their chromosomes have properly aligned or not. Cells that exit with unaligned chromosomes will become aneuploid and die because they lack chromosomes that are essential for life. Inhibition of the mitotic checkpoint may also be used in conjunction with conventional anti-microtubule drugs to enhance cell killing. One of the responses of tumors when they are exposed to microtubule poisons is to activate the mitotic checkpoint. If tumor cells are able to mount a robust checkpoint response, they may remain blocked in mitosis until the drug is metabolized, at which time they resume progression through the cell cycle. While this response may be difficult to document in patients, it is feasible to test whether inhibition of the mitotic checkpoint will sensitize cells in culture to existing microtubule poisons.

The search for inhibitors of the mitotic checkpoint is now possible because many proteins that are essential for this process have been identified. Pioneering studies in budding yeast identified six evolutionarily conserved proteins, Mad1, Mad2 Mad3, Bub1, Bub3 and Mps1, that are essential for cells to arrest in mitosis in the presence of spindle damage.[22-24] In metazoans, many of these proteins have been shown to localize to kinetochores where they are postulated to monitor the status of microtubule attachments and the amount of kinetochore tension that develops as a consequence of opposing poleward forces.[19] Although the precise role of each of these proteins in the checkpoint pathway remains to be clarified, it is clear that all of them are essential for cells to arrest in mitosis in response to unattached kinetochores. Given that MPS1, BUB1 and the Mad3-related BUBR1 are all protein kinases, it is likely that they are part of a kinase cascade that initiates at an unattached kinetochore and is amplified throughout the cell to inhibit the ubiquitin ligase Anaphase Promoting Complex. These kinases are therefore prime targets for drug development given the existence of large libraries of compounds that were designed for identifying kinase inhibitors. In contrast to the protein kinases, the biochemical properties of the Bub3, Mad1 and Mad2 checkpoint proteins remain unknown as their primary sequence do not reveal distinctive motifs. As these proteins are known to form complexes with each other and some of the checkpoint kinases, it may be possible to screen for compounds that disrupt these protein interactions. This is a feasible strategy given that overexpression of a specific fragment of Mad1 will sequester the endogenous Mad2 so that it cannot form complexes with other checkpoint proteins in the cell.[25] As a consequence, cells overexpressing the Mad1 fragment are unable to arrest in mitosis.

A KINETOCHORE ASSEMBLY IN HUMAN CELLS?

The observation that kinetochores are only visible in mitosis suggests that this structure undergoes cell cycle dependent assembly and disassembly. This is supported by the existence of proteins that are only detected at kinetochores in mitosis. Kinetochore proteins can be categorized as constitutive or transient based on their temporal patterns of localization in human cells. Proteins such as CENP-A, CENP-B, CENP-C, CENP-G, CENP-H, CENP-I and hMIS12 belong to the constitutive class because they are localized to kinetochores throughout the cell cycle (strictly speaking, they are associated with pre-kinetochores during interphase). A larger group of proteins are only transiently detected at kinetochores during mitosis (Table 1). Thus, the constitutive kinetochore proteins form a core to which transient kinetochore proteins are recruited to in a cell-cycle

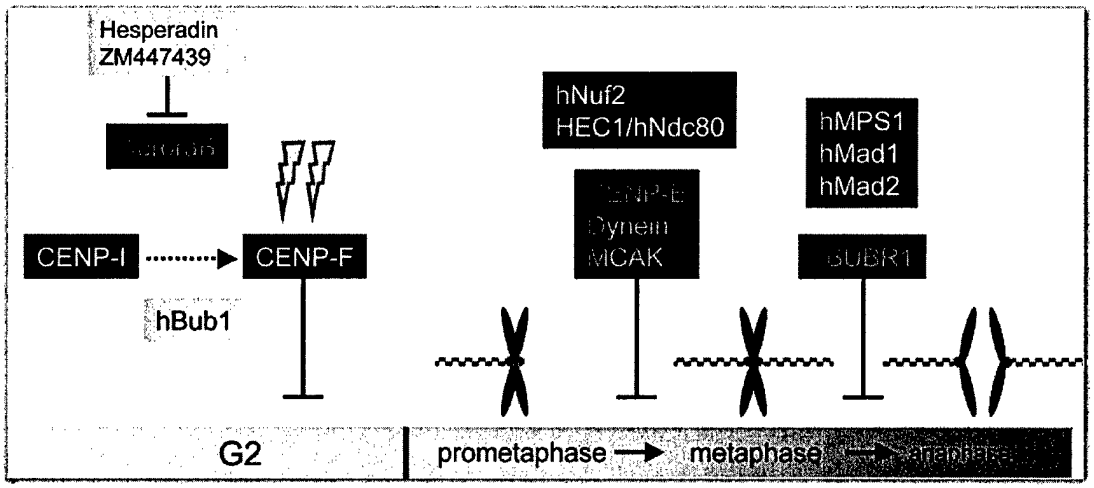

Figure 1. Schematic diagram of the kinetochore assembly pathway and potential sites for drug intervention. Kinetochore proteins are depicted according to their approximate temporal pattern of localization. Proteins that lie along the same assembly pathway are depicted in the same color. The exception is that the localization of Mad1 and Mad2 are known to be dependent on CENP-I and HEC1/hNdc80 but it is unknown whether these proteins define two converging pathways or lie along the same pathway. Critical transition points in the cell cycle are defined by CENP-F, the microtubule motors, and checkpoint proteins. These sites may be targets for drugs or radiation.

dependent manner. For example, the kinesin-like motor protein, CENP-E, accumulates in the cytoplasm during interphase and is not detected at kinetochores until the onset of mitosis.[26] Interestingly, not all transient kinetochore proteins are detected at kinetochores at the same time. A careful comparison of the temporal localization pattern of various members of the transient family of kinetochore proteins revealed a distinct order of assembly (Fig. 1). For example, CENP-F is a protein that is uniformly distributed in nuclei of cells in the G_2 phase of the cell cycle. It is first detected at kinetochores during late G_2 and thus precedes the appearance of CENP-E at kinetochores.[27] Similarly, the hBUB1 checkpoint kinase is also first detected at kinetochores in G_2. Remarkably, its appearance at kinetochores precedes CENP-F.[28] These observations suggest that proteins such as hBUB1 and CENP-F may define discrete segments of G_2 and that this phase of the cell cycle may specify kinetochore assembly much like DNA replication defines S phase.

The recent characterization of human CENP-I has revealed some additional complexities to the kinetochore assembly process.[29] CENP-I is a constitutive kinetochore protein that exhibits limited sequence similarity with Mis6 and Ctf3 kinetochore proteins in fission and budding yeast, respectively. Unlike Mis6 which is essential for the loading of the histone H3 variant, CENP-A, onto centromeres in fission yeast,[30] this relationship does not appear to be conserved in humans, chickens and budding yeast.[29,31,32] In Hela cells. CENP-I was shown to be essential for CENP-F, Mad1 and Mad2 to assemble onto kinetochores. The failure of these proteins to assemble onto kinetochores is not due to gross disruption of this structure as other transient kinetochore proteins such as CENP-E, dynein, hBUB1 and hBUBR1 were not affected by the loss of CENP-I. These data show that kinetochore assembly does not follow a single linear pathway but is branched. Each branch may specify a discrete functional domain within the kinetochore.

Studies have also revealed that the kinetochore assembly pathway may be monitored by the checkpoint. Hela cells depleted of CENP-I were found to be delayed in G_2 at a stage when CENP-F was still distributed uniformly in the nucleus.[29] This delay was estimated to last for approximately three hours before cells resumed their progression into mitosis. It is unknown at this time how kinetochore assembly is monitored. Interestingly, kinetochore assembly appears to be linked to the DNA damage checkpoint. Hela cells that were exposed to ionizing radiation or etoposide were found to accumulate at a discrete stage in G_2 prior to when CENP-F assembled onto kinetochores.[33] The molecular basis for this connection is not clear but it is likely that the DNA damage response inhibits key cell-cycle regulators whose activities are required for CENP-F to assemble onto the growing kinetochore. It is perhaps not a coincidence that cells depleted of CENP-I are delayed at the same point in G_2 as the cells with DNA damage. The combined results strongly suggests that the assembly of CENP-F onto kinetochores might represent a critical point in G_2 where it is subject to checkpoint intervention. It is possible that the assembly of CENP-F onto kinetochores may represent a key transition point where cells commit themselves to proceed into the next phase of the cell cycle.

DISRUPTING KINETOCHORE ASSEMBLY

We believe that the ordered assembly of proteins onto kinetochores may serve two purposes (Fig. 1). The first is to provide a mechanism to ensure that a kinetochore is properly assembled. Thus, the successful assembly of one protein may be critical for the subsequent assembly of proteins onto the growing kinetochore. The second reason is that the order of assembly reflects the spatial organization of discrete functional domains to kinetochores. Thus, the disruption of kinetochore assembly may be a strategy that effectively inhibits multiple proteins that provide critical functions to kinetochores. This approach may be an important alternative to conventional approaches to search for inhibitors of kinases or molecular motors. In those cases, it may be difficult to identify inhibitors with the desired specificity. This is a serious concern as there are a large number of protein kinases and kinesin-like proteins that provide critical functions to non-dividing cells. By contrast inhibitors of the putative structural proteins may be more specific as they must act by disrupting interactions with other proteins.

Recent reports of the human kinetochore proteins hNuf2, HEC1/hNdc80 help to illustrate the utility of inhibiting kinetochore assembly.[34,35] Nuf2 and Ndc80 were originally identified in budding yeast as part of a tetrameric complex that includes Spc25 and Spc25.[36] In human cells, both hNuf2 and HEC1/hNdc80 localize to kinetochores consistent with their yeast counterparts. Hela cells depleted of hNuf2 and HEC1/hNdc80 accumulate in mitosis because their chromosomes failed to attach to the spindle.[34,35] Kinetochores in cells depleted of HEC1/hNdc80 lack detectable levels of the checkpoint proteins Mad1, Mad2, Mps1 and reduced amounts of hBUB1.[34] Similarly, kinetochores depleted of hNuf2 also show reduced levels of Mad2 and hBubR1[35] (Jablonski and Yen, unpublished data). Although the long-term fate of the HEC1/hNdc80 depleted cells is not known, the hNuf2 depleted cells arrest in mitosis but eventually die[35] (Jablonski and Yen, unpublished data). Kinetochores lacking hNuf2 or HEC1/hNdc80 retained CENP-E and dynein but the localization of other microtubule binding proteins is not known. As hNuf2 and HEC1/hNdc80 are not known to interact directly with microtubules, the mechanism by which they specify attachment of chromosomes to the spindle is unclear. Regardless of how loss of these proteins disrupt chromosome segregation, the outcome of inhibiting of these proteins is very similar to that when dividing cells are treated with microtubule poisons.

A significant obstacle in searching for inhibitors to proteins such as hNuf2 and HEC1/hNdc80 is that it is difficult to develop in vitro assays without the knowledge of their biochemical activities. However, it may be possible to take advantage of the fact that these proteins and the pathway they specify are conserved in yeast. The strategy to use yeast as a tool to identify drugs that target pathways that are conserved between yeast and humans was developed a number of years ago as part of the NCI sponsored Seattle Project.[37] As yeast grow rapidly, compounds can be simply assayed by monitoring their effects on growth. As the assay can be conducted in microtiter plates, it facilitates automation and large scale screening efforts. As HEC1/hNdc80 has been reported to complement ndc80 mutant yeast, a screen could in principle be designed to identify inhibitors of the human homolog.

INHIBITORS OF KINETOCHORE PROTEINS

Farnesyl transferase inhibitors (FTI) which were designed to inhibit cancer cells that express constitutively active Ras mutants have since been found to interfere with mitosis in some cell lines.[38-40] It is interesting that both CENP-E and CENP-F are farnesylated and thus raise the possibility that they may be targets for inhibition by FTI's.[38] However, two studies using different FTI's (FTI-2153 and SCH66336) showed that the localization of both proteins to kinetochores was unaffected by these drugs.[38,39] Nevertheless, mutation of the invariant C in the CAAX motif prevented CENP-F from accumulating at kinetochores.[40] These results suggest that the localization of CENP-F and possibly CENP-E is dependent on farnesylation. In the presence of FTI's, CENP-E and CENP-F may undergo alternate modifications such as geranylgeranylations that may be sufficient for their localization to kinetochores. Regardless of the effects of FTI's on CENP-E and CENP-F, the FTI-253 was shown to block the lung cancer cell lines A-549 and Calu-1 arrest in mitosis.[39] The arrest was attributed to the failure to establish a bipolar spindle. This observation suggests that FTI's may have additional targets in the centrosome.

Aurora kinase plays a critical role in mitosis as it is important for spindle formation and attachment of chromosomes to the spindle.[41] Mammalian cells express three versions of aurora. Aurora A and B are localized to centrosomes and kinetochores respectively. Aurora C expression appears to be restricted to testes so its role in mitosis is not certain. The Aurora kinases were targeted for drug discovery as their expression was elevated in many cancer cell lines.[42] Two recent studies report on the characterization of inhibitors of aurora kinase in mammalian cells.[43,44] Hesparadin and ZM447439 are kinase inhibitors that appear to be fairly specific for aurora kinases in vitro. Although both inhibitors are equally effective in inhibiting Aurora A and B in vitro, their effects on cells suggest Aurora B maybe more sensitive in vivo. Aurora B is localized to kinetochores where it is believed to facilitate attachment of microtubules.[45] In yeast, the aurora-related, Ipl1 kinase is believed to ensure that kinetochores do not become attached to the same pole.[46] Such "syntelic" attachments would escape detection by the checkpoint because the kinetochores are saturated with microtubules. If uncorrected, chromosomes would remain attached to a single pole and undergo non-disjunction. Ipl1 is thought to be able to detect monopolar attached chromosomes because their kinetochores do not develop sufficient tension. Ipl1 is therefore thought to stimulate the release of microtubules from kinetochores that are not under tension. This has important implications for the anti-cancer drug taxol as this drug is known to suppress microtubule dynamics and thus prevent the establishment of tension at kinetochores. The absence of tension is believed to be the mechanism by which taxol treated cells remain arrested in mitosis despite the fact that they have a fully formed spindle that is attached to chromosomes. This contrasts with drugs such as vinblastine and colchicine which arrest cells in mitosis because kinetochores lack microtubule attachments. Remarkably, inhibition of Aurora B with Hesparadin and ZM447439 abrogated the taxol mediated arrest and caused cells to exit. In contrast, their effects are much lower in affeting the ability of microtubule destabilizing drugs such as nocodazole from arresting cells in mitosis.[43,44] These results suggest that inhibition of Aurora B might sensitize tumor cells to treatment with taxol but not the vincalkaloids.

CONCLUSIONS

Studies over the past decade have revealed a highly conserved mechanism by which eucaryotic cells accurately segregate their chromosomes. These studies have identified many molecular components that are essential for this process. As such, these proteins should be ideal candidates for the development of highly specific anti-mitotic drugs for the treatment of cancer (Fig. 1). Although this review has focused on kinetochores, there are many proteins that are involved in spindle assembly that also qualify as targets for drug discovery. Thus, the future challenge is no longer target identification but target selection.

Reference

1. Hadfield JA, Ducki S, Hirst N, McGown AT. in Progress in Cell Cycle Research (ed. Meijer, L., Jezequel, A., and Roberge, M.) 309-325 (Editions "Life in Progress", Roscoff, France, 2003).
2. Yusuf RZ, Duan Z, Lamendola DE, Penson RT, Seiden MV. Paclitaxel resistance: molecular mechanisms and pharmacologic manipulation. Curr Cancer Drug Targets 2003; 3:1-19.
3. Rieder CL. The formation, structure, and composition of the mammalian kinetochore and kinetochore fiber. Int Rev Cytol 1982; 79:1-58.
4. Cleveland DW, Mao Y, Sullivan KF. Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell 2003; 112:407-21.
5. Maney T, Ginkel LM, Hunter AW, Wordeman L. The kinetochore of higher eucaryotes: a molecular view. Int Rev Cytol 2000; 194:67-131.

6. Rieder CL, Salmon ED. The vertebrate cell kinetochore and its roles during mitosis. Trends Cell Biol 1998; 8:310-8.
7. Yen TJ, Li G, Schaar BT, Szilak I, Cleveland DW. CENP-E is a putative kinetochore motor that accumulates just before mitosis. Nature 1992; 359:536-9.
8. Steuer ER, Wordeman L, Schroer TA, Sheetz MP. Localization of cytoplasmic dynein to mitotic spindles and kinetochores. Nature 1990; 345:266-8.
9. Pfarr CM, et al. Cytoplasmic dynein is localized to kinetochores during mitosis. Nature 1990; 345:263-5.
10. Wordeman L, Mitchison TJ. Identification and partial characterization of mitotic centromere-associated kinesin, a kinesin-related protein that associates with centromeres during mitosis. J Cell Biol 1995; 128:95-104.
11. Dujardin D, et al. Evidence for a role of CLIP-170 in the establishment of metaphase chromosome alignment. J Cell Biol 1998; 141:849-62.
12. Tirnauer JS, Canman JC, Salmon ED, Mitchison TJ. EB1 Targets to Kinetochores with Attached, Polymerizing Microtubules. Mol Biol Cell 2002; 13:4308-1.
13. Lemos CL, et al. Mast, a conserved microtubule-associated protein required for bipolar mitotic spindle organization. Embo J 2000; 19:3668-82.
14. Schaar BT, Chan GKT, Maddox P, Salmon ED, Yen TJ. CENP-E function at kinetochores is essential for chromosome alignment. J Cell Biol 1997; 139:1373-82.
15. McEwen BF, et al. CENP-E is essential for reliable bioriented spindle attachment, but chromosome alignment can be achieved via redundant mechanisms in mammalian cells. Mol Biol Cell 2001; 12, 2776-89.
16. Putkey FR, et al. Unstable kinetochore-microtubule capture and chromosomal instability following deletion of CENP-E. Dev Cell 2002; 3:351-65.
17. Hunter AW, et al. The kinesin-related protein MCAK is a microtubule depolymerase that forms an ATP-hydrolyzing complex at microtubule ends. Mol Cell 2003; 11:445-57.
18. Desai A, Verma S, Mitchison TJ, Walczak CE. Kin I kinesins are microtubule-destabilizing enzymes. Cell 1999; 96:69-78.
19. Chan GK, Yen TJ. in Progress in Cell Cycle Research (ed. Meijer, L., Jezequel, A., and Roberge, M.) 431-439 (Editions "Life in Progress", Roscoff, France, 2003).
20. Musio A, et al. Inhibition of BUB1 Results in Genomic Instability and Anchorage-independent Growth of Normal Human Fibroblasts. Cancer Res 63, 2855-63 (2003).
21. Dobles M, Liberal V, Scott ML, Benezra R, Sorger PK. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. Cell 2000; 101:635-45.
22. Li R, Murray AW. Feedback control of mitosis in budding yeast. Cell 1991; 66:519-31.
23. Hoyt MA, Totis L, Roberts BT. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. Cell 1991; 66:507-17.
24. Weiss E, Winey M. The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. J Cell Biol 1996; 132:111-23.
25. Canman JC, et al. Anaphase onset does not require the microtubule-dependent depletion of kinetochore and centromere-binding proteins. J Cell Sci 2002; 115:3787-95.
26. Yen TJ, et al. CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. Embo J 1991; 10:1245-54.
27. Liao H, Winkfein RJ, Mack G, Rattner JB, Yen TJ. CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. J Cell Biol 1995; 130:507-18.
28. Jablonski SA, Chan GK, Cooke CA, Earnshaw WC, Yen TJ. The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. Chromosoma 1998; 107:386-96.
29. Liu ST, et al. Human CENP-I specifies localization of CENP-F, MAD1 and MAD2 to kinetochores and is essential for mitosis. Nat Cell Biol 2003; 5:341-5.
30. Saitoh S, Takahashi K, Yanagida M. Mis6, a fission yeast inner centromere protein, acts during G1/S and forms specialized chromatin required for equal segregation. Cell 1997; 90:131-43.
31. Nishihashi, A. et al. CENP-I is essential for centromere function in vertebrate cells. Dev Cell 2002; 2:463-76.
32. Measday V, et al. Ctf3p, the Mis6 budding yeast homolog, interacts with Mcm22p and Mcm16p at the yeast outer kinetochore. Genes Dev 2002; 16:101-13.
33. Fletcher L, Yen TJ, Muschel RJ. DNA damage in HeLa cells induced arrest at a discrete point in G2 phase as defined by CENP-F localization. Radiat Res 2003; 159:604-11.
34. Martin-Lluesma S, Stucke VM, Nigg EA. Role of hec1 in spindle checkpoint signaling and kinetochore recruitment of mad1/mad2. Science 2002; 297:2267-70.
35. DeLuca JG, Moree B, Hickey JM, Kilmartin JV, Salmon ED. hNuf2 inhibition blocks stable kinetochore-microtubule attachment and induces mitotic cell death in HeLa cells. J Cell Biol 2002; 159:549-55.
36. Wigge PA, Kilmartin JV, The Ndc80p complex from Saccharomyces cerevisiae contains conserved centromere components and has a function in chromosome segregation. J Cell Biol 2001; 152:349-60.
37. Simon JA, Yen TJ. in Methods in Molecular Biology (ed. El-Deiry, W. S.) 555-576 (Humana Press Inc., Totowa, New Jersey, 2003).
38. Ashar HR, et al. Farnesyl transferase inhibitors block the farnesylation of CENP-E and CENP-F and alter the association of CENP-E with the microtubules. J Biol Chem 2000; 275:30451-7.
39. Crespo NC, Ohkanda J, Yen TJ, Hamilton AD, Sebti SM. The farnesyltransferase inhibitor, FTI-2153, blocks bipolar spindle formation and chromosome alignment and causes prometaphase accumulation during mitosis of human lung cancer cells. J Biol Chem 2001; 276:16161-7.
40. Hussein D, Taylor SS. Farnesylation of Cenp-F is required for G2/M progression and degradation after mitosis. J Cell Sci 2002; 115:3403-14.
41. Shannon KB, Salmon ED. Chromosome dynamics: new light on Aurora B kinase function. Curr Biol 2002; 12:R458-60.
42. Bischoff JR, Plowman GD. The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. Trends Cell Biol1999; 9:454-9.
43. Ditchfield C, et al. Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J Cell Biol 2003; 161:267-80.
44. Hauf S, et al. The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. J Cell Biol 2003; In Press.
45. Adams RR, Carmena M, Earnshaw WC. Chromosomal passengers and the (aurora) ABCs of mitosis. Trends Cell Biol 2001; 11:49-54.
46. Tanaka TU, et al. Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. Cell 2002; 108:317-29.
47. Waters JC, Chen RH, Murray AW, Salmon ED. Localization of Mad 2 to kinetochores depends on microtubule attachment, not tension. J Cell Biol 1998; 141:1181-91.